PRELIMINARY STUDIES OF THE EFFECTS OF DICHROMATE ION ON SURVIVAL AND OXYGEN CONSUMPTION OF *DAPHNIA PULEX* (L.)

BY

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INTRODUCTION

Hexavalent chromium compounds (chromates and dichromates) are utilized in many industries, especially metal plating, anodyzing aluminium, leather tanning, and manufacture of such products as paints, dyes, explosives, ceramics, and paper (Besselièvre, 1969; McKee & Wolf, 1963). In addition, the resistance of dichromate to corrosion makes it an important addition to cooling water in many industries. Because hexavalent chromium occurs in many industrial effluents, its effects on organisms is of interest.

The USPHS Drinking Water Standards of 1946 and 1962 set a mandatory limit of 0.05 mg/l for hexavalent chromium (McKee & Wolf, 1963). Although the standard is quite low compared to toxic levels for mammals and fish (Mertz, 1969), microcrustaceans are sensitive to extremely low levels of hexavalent chromium, and may not be protected by present standards. Because microcrustaceans form an essential link in the aquatic food chain, both as primary consumers (usually) and as prey for a wide variety of fish and other secondary consumers, their survival as individuals and as species should be a consideration in determining water quality standards. Both descriptions of toxic effects of chromium compounds on microcrustaceans and information on their mechanisms of action are needed.

Toxicity levels of hexavalent chromium compounds on *Daphnia magna* Straus and *Gammarus pulex* (L.) range from 0.05 to 1.4 mg/l (table I). Effects on *D. pulex* (L.) and other microcrustaceans apparently have not been tested. The present preliminary study was undertaken to determine the effect of sodium dichromate ($Na_2Cr_2O_7$) on survival of *Daphnia pulex,* and to observe effects of age and density of *D. pulex* on response to dichromate. In addition, studies of oxygen consumption were conducted to elucidate some of the interactions among respiration, density, and dichromate toxicity.

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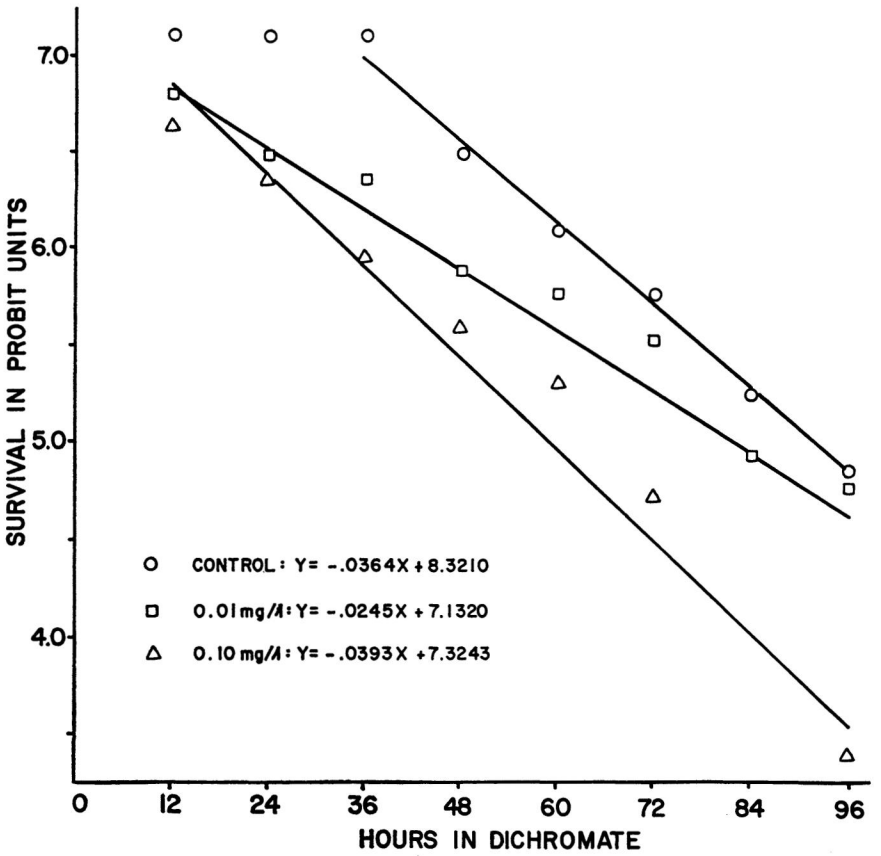

Fig. 5. Regression of *Daphnia pulex* survival (in probit units) against time at three concentrations of $Na_2Cr_2O_7$. Animals were six days old at Hour O, and were tested at a density of 1 *Daphnia*/2 ml. Each line represents the survival of 57 animals. Lines extend only through points used in the regressions analysis.

TABLE IV

Comparison of homogeneity of position of regression lines using Kolmogorov-Smirnov two-sample tests (Siegel, 1956). The test is based on absolute numbers of dead *Daphnia pulex* at each of two densities.

a. Comparison of concentrations at a density of 1 *Daphnia*/2 ml. $D = \max [s_{n1}(x)-s_{n2}(x)]$

Comparison	D	Signif.
cont. vs 0.1 mg/l	28/57 = .4912	$p<.001$
cont. vs 0.01 mg/l	7/57 = .1228	n.s.
0.01 vs 0.1 mg/l	22/57 = .3860	$p<.001$

b. Comparison of concentrations at a density of 1 *Daphnia*/4 ml.

Comparison	K_d	Signif.
cont. vs 0.1 mg/l	10/24	$p<.05$
cont. vs 0.01 mg/l	6/24	n.s.
0.01 vs 0.1 mg/l	11/24	$p<.05$

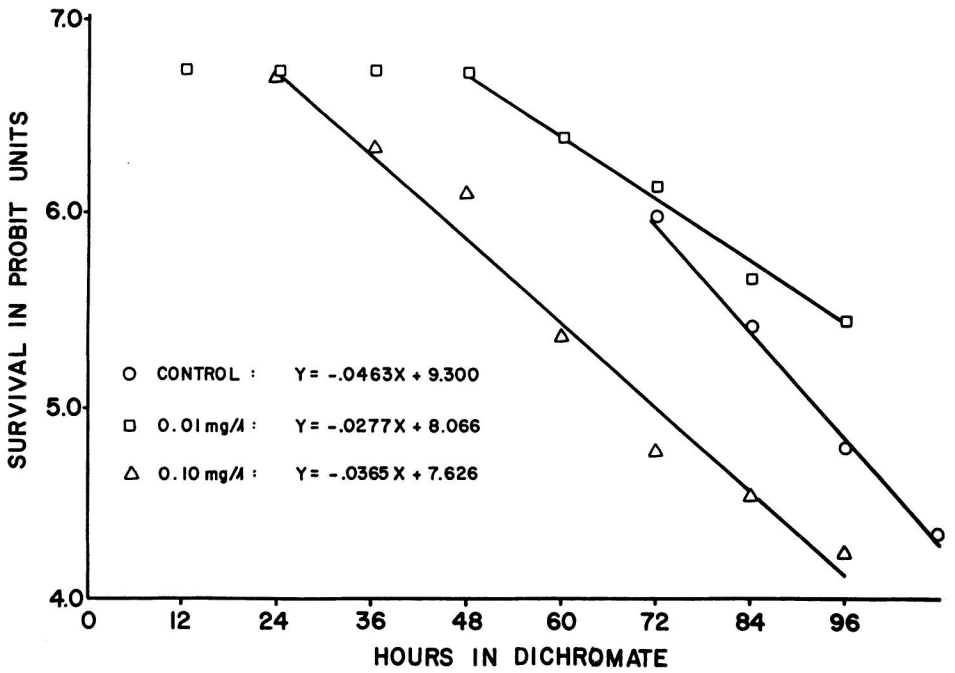

Fig. 6. Regression of *Daphnia pulex* survival (in probit units) against time at three concentrations of $Na_2Cr_2O_7$. Animals were six days old at Hour O, and were tested at a density of 1 *Daphnia*/4 ml. Each line represents the survival of 24 animals. Lines extend only through points used in the regression analysis.

TABLE V

Values of Student's t for comparisons of oxygen consumptions of dichromate-exposed and normal *Daphnia pulex* at two densities. C and D refer to control and dichromate groups, respectively; 8 and 16, to numbers of animals per 60 ml bottle. Numbers in parentheses indicate total numbers of animals measured. The dichromate groups were exposed to 0.01 mg/l $Na_2Cr_2O_7$; all groups were allowed to consume oxygen for 40 hours. Oxygen consumption measurements are the averages of 10 replicates per group.

μg O_2 consumed/animal/hour

	C-8(80)	C-16(160)	D-8(80)	D-16(160)	s	t	p
D-8 vs D-16			.2881	.2227	.1103	1.779	n.s.
C-8 vs C-16	.1487	.1029			.0495	2.776	$p<.05$
D-8 vs C-8	.1487		.2881		.1103	3.791	$p<.01$
D-16 vs C-16		.1029		.2227	.1075	3.343	$p<.01$

TABLE VI

Values for Student's t for comparisons of weight-specific oxygen consumptions of dichromate-exposed and normal *Daphnia pulex* at two densities. The experiment is the same one as described in table V.

	μg O_2 consumed/mm^3/hour						
	C-8(80)	C-16(160)	D-8(80)	D-16(160)	s	t	p
D-8 vs D-16			.1273	.0857	.0484	2.578	$p<.05$
C-8 vs C-16	.0691	.0598			.0110	2.536	$p<.05$
D-8 vs C-8	.0691		.1273		.0484	3.607	$p<.01$
D-16 vs C-16		.0598		.0857	.0110	7.064	$p<.001$

DISCUSSION

Three groups of factors affect the toxicity of a pollutant to a particular species: 1) the characteristics of the pollutant itself, such as physical and chemical properties and concentration, 2) the characteristics of the animal, including genetics, physiological state, activity and previous history, and 3) the characteristics of the external environment, for example temperature, light, pH and other organisms. The ultimate response of any organism to a pollutant is determined by the interaction of all factors. In the present experiments three factors — concentration of dichromate, age of *Daphnia* and density of *Daphnia* — were chosen for several reasons. First, they represent each of the three groups of factors described above. Secondly, they seemed likely to illustrate intraspecific variation in the toxic response. And finally, the study of several factors served as a model for attempting to understand more complex interactions between animals and their environments.

It is impossible to discuss the effects of a toxin on individual organisms without relating the effects to the interactions mentioned above. The following interactions will therefore be considered: concentration versus concentration, concentration versus density, and concentration versus age (or stage in life cycle). Oxygen consumption as an illustration of a possible mechanism in the toxicity response will be discussed in relation to density and age of *Daphnia.*

According to Skidmore (1964), "the survival time of aquatic animals exposed to a poison is inversely related to the concentration of poison." In the present data higher death rates generally occurred at higher concentrations. Variations and lack of significance may be due to small increments between concentrations tested or small differences in toxicity resulting from the extremely low concentrations tested. Another reason may be the biochemical utilization of low levels of chromium. The data indicate that *Daphnia* show no adverse reaction to very low levels of dichromate (tables II, IV). In some cases (fig. 6) a low concentration of dichromate may even have a slight (though nonsignificant) positive effect on *Daphnia* sur-

vival. Concentration of radiochromium by crayfish has been reported (Foster, 1963). Content of chromium in the gills, muscles, midgut gland, carapace, and blood of the crab *Podophthalmus vigil* (Fabricius) were fairly constant during the molt cycle (Sather, 1966). Sather suggested implication of chromium in induction of calcification of the integument and in action as a "glucose tolerance factor", increasing the utilization of glycogen by the epidermal cells. Therefore, chromium is concentrated and possibly utilized by crustaceans, although no evidence is presently available for microcrustaceans.

In the first set of experiments a slight but consistent variation from linearity in the time-mortality curves occurred at all concentrations. This variation is a repeating pattern of a slow rate followed by an increasing rate of mortality (figs. 3, 4). Although my conclusions about *Daphnia* survival are provisional because of the relatively small number of animals used, a possible explanation for the "stair-step" pattern of survival can be suggested. In any fairly large group of animals, individual differences in susceptibility probably tend to fall together in groups. At the beginning of the time course, there will necessarily be a lag in mortality until sufficient toxin has been absorbed by the animals. This lag will be greater or lesser depending on the general physiological condition of the animals, rate of acclimation, and concentration of the toxin. As sufficient toxin is absorbed, there is an increase in the death rate, with the most susceptible group of animals dying first. The lag in deaths near the middle of the time course is in part a natural result of the earlier high death rate — fewer sensitive animals are left to die during this time. In addition, the less sensitive animals have had time to become somewhat acclimated to the toxin. After the plateau, which lasts anywhere from 36 to 72 hours, another less sensitive group dies, again causing an increased death rate. Presumably the pattern continues until 100% mortality occurs.

The repeating pattern is most evident in the first set of experiments, probably because of the high density at which the experiments were conducted. In the second set of experiments, where two lower densities were used, the results indicate the beginnings of such a pattern. However, the pattern appears to be attenuated, i.e., the effects of the toxin are spread out over a longer time span. This lag in the effect of dichromate on survival is especially evident at the lowest density (fig. 6), where no deaths occur until 24 hours at 0.10 mg/l and 48 hours at 0.01 mg/l of dichromate.

Comparison of regression coefficients at the three densities is also important in describing the toxic response. Table IIIc indicates no significant differences in regression coefficients at the two lower densities. However, inspection of regression coefficients suggests that, at 0.01 mg/l of dichromate, the high density approximately doubles the rate of mortality in 6-day-old animals (figs. 2, 5, 6). At 0.10mg/l, however, the slope is probably not significantly different from those at 2 and 4 ml/*Daphnia*. This may indicate that somewhere between 0.01 and 0.10 mg/l a threshold is reached where any further decrease in density does not appreciably increase the chances of survival. Unfortunately, at a concentration of 0.05 mg/l only one density was tested.

The theory of differential susceptibility (Child, 1915, in Fowler, 1931) states that "the animal or part of an animal which has the highest metabolic rate is the most susceptible to a great variety of injurious agents, both chemical and physical." The related theory of differential acclimation states that "if these injurious agents are not too fatal, so that there is some acclimation, the organism or part of the organism with the highest metabolic rate will acclimate most rapidly and survive longer than one with a slower metabolic rate."

Metabolic rate, which affects the response of animals to toxic substances, is in turn affected by animal density. There is a tendency for grouping (i.e., increased density) to lower the rate of oxygen consumption per animal (Allee, 1951; Fowler, 1931). This tendency is substantiated by the results of the present experiment (tables V, VI), although significance levels are low. Application of Child's theories suggests that a larger group, in which individuals have lowered rates of oxygen consumption, will be more resistant to high concentrations of toxin. On the other hand, when exposed to relatively weak, slow-acting toxins, animals with higher oxygen consumptions (small groups or singles) will be favored.

In the present experiments concentrations of 0.01 mg/l and below could with little argument be classed as "slowacting" ("low" concentrations), since the effect of these concentrations on survival cannot be distinguished statistically from controls. Probably 0.05 mg/l could also be classed as slow-acting. Concentrations of 0.10 mg/l and above act considerably more rapidly (figs. 2, 4, 5). Overall, then, we might expect that lower densities, with higher oxygen consumptions per animal, would be more conducive to survival than higher densities. This is indeed the result seen in the survival experiments. Unfortunately, the two highest concentrations (0.05 and 0.10 mg/l) were measured at only one density. More experiments should be conducted at these levels, since it is likely that the area of concentrations between 0.01 and 0.10 mg/l of sodium dichromate is a "threshold" area in which the physiological responses of adult *D. pulex* undergo a change.

There is an apparent difference in slopes between 6- and 12-day-old animals exposed to dichromate (fig. 2). However, the significant differences between controls at the two ages (table IIc) indicates that the trend toward greater resistance of the older animals is merely an artifact, and that given ideal conditions the 6-day-olds would have appeared as hardy as the 12-day-olds.

MacArthur & Baillie (1929) state that in *D. magna* heart-beat rate, a measure of metabolism, is slow in new-hatched young, reaches a maximum at 7 days, and drops slowly until death. Since *D. pulex* also has 4 to 6 pre-adult instars, the first young are produced at 5 to 7 days. Therefore, animals at 6 days of age would be reproductively mature and would presumably have the highest respiration rate of their lives. Because of both size and age, 6-day-olds would be expected to have a higher respiration rate. Assuming the accuracy of Child's (1915) and Allee's (1951) theory concerning oxygen consumption effects on survival, one would expect that the 6-day-olds would acclimate faster, therefore be less susceptible to the low concentrations tested than would 12-day-olds. If any difference in susceptibility occurs, however, it is the opposite effect.

Possibly there is enough size difference between 6- and 12-day-olds to compensate for the respiration effect. According to Hassenteufel et al. (1963) one of the factors upon which adsorption depends is the ratio between the volume of the solution and the surface area of the solid in question. With a larger surface area, the older animals would adsorb more dichromate, leaving a smaller amount available in the medium. However, the same effect should be caused by increasing the density, resulting in higher densities having a survival advantage. As indicated earlier, lower densities have a greater survival advantage. Obviously, the interactions between density, age, and concentration of dichromate are complex and are not well-elucidated by the present experiments.

Qualitative observations showed little reproductive activity occurring during exposure to dichromate in the survival experiments. In some cases young were hatched during the experiment. Apparently the eggs were formed in the ovaries before the adults were exposed to dichromate, and were released into the brood chambers soon after exposure. The young were not removed from the solutions, and although quite active at first, they usually died before (or perhaps during) the first instar. Perhaps Anderson's (1948) comment that *D. magna* are more susceptible to $ZnCl_2$ at the time of molting, when the exoskeleton is soft and more easily penetrated by zinc, applies here.

In the oxygen consumption experiments a considerably different situation in regard to reproduction was found. All animals chosen for oxygen consumption experiments were six days old and were not carrying eggs in the brood pouches. However, by the end of the experiments, 35.3 (at a density of 16 *Daphnia*/60 ml) and 40.9% (at 8 *Daphnia*/60 ml) of the animals exposed to dichromate had eggs or well formed young in the brood pouches or had already hatched young. Although animals were chosen randomly for the dichromate and control groups, only 6.0% (at 16 *Daphnia*/60 ml) and 8.6% (at 8 *Daphnia*/60 ml) of the control animals had young in the brood pouches. The resultant oxygen consumption figures for the dichromate-exposed animals were approximately twice as high as oxygen consumptions for control animals. Because of the presence of young in the experimental group, it is not possible to state with certainty that the oxygen consumption rate is increased by exposure to dichromate. However, the presence of young in itself suggests a significant difference in response of the two groups. Eggs in the dichromate groups obviously were released from the ovaries and developed into young in a shorter time than in the control groups, indicating that a higher oxygen consumption must have prevailed in the dichromate-exposed animals. There is no evidence to indicate whether increasing the concentration of dichromate would continue to result in increased oxygen consumption.

As indicated by the high death rate among control animals in all survival experiments, some factor or factors other than dichromate acted to cause mortality. It is important to attempt to understand these factors. Several possibilities can be suggested. Survival of controls was lowest at a density in 1 *Daphnia*/ 1 ml (17.5% and 25% at termination of the experiments). At the lower densities, survival at

96 hours was considerably higher (41.7%, 43.9%). A relationship is thus suggested between density and the unknown stress factors. Factors which might be affected by density, especially in the small test containers used, include oxygen concentration and concentration of toxic metabolites. A simple calculation can be done to determine whether lowering of oxygen concentration was great enough to cause a stress on control animals. Oxygen, at a total pressure of 760 mm (assumed) and a temperature of 21°C (actual), has a solubility of 0.004252 g (or 4.252 mg) in 100 g (ml) of water. Thus there is 0.34016 mg O_2/8 ml H_2O. The highest oxygen consumption of control animals tested was 0.1487 μg (or 0.0001487 mg) O_2/animal/hour (table VI). At this rate of utilization, 8 animals (the number per replicate at the highest density) in 96 hours would use 0.1142 mg O_2, or approximately one-third of the O_2 in an 8-ml saturated test sample. Oxygen consumption per animal decreases with increasing density (Fowler, 1931; Allee, 1951; present paper). However, since the oxygen consumption measurement used above reflects the rate at the lowest density multiplied by the number of animals at the highest density, it would be expected that, given the decreasing rate at higher densities, even less than 0.1142 mg O_2 would be used. Thus, decreased O_2 tensions would appear not to have been a factor in control deaths.

Minor errors in the above calculations could be due to pressure variations and to the fact that calculations were based on distilled water rather than *Daphnia* medium. A more serious possibility is the difference in volume of medium in survival and O_2 consumption tests. Survival tests were run in 8 ml of medium; O_2 consumptions in 60 ml per sample. Zeiss (1963) discusses the stress caused by confinement in a small space; *Daphnia magna*, at densities of 5 animals per 1.2 ml and 5 animals per 0.6 ml, consumed up to 2½ times as much oxygen as when placed in 60 ml of medium. Animals were confined within 60 ml bottles which were stirred constantly, eliminating O_2 depletion and build-up of waste products as stress factors. Although present densities were not as high the possibility exists that, because of smaller containers, O_2 consumptions in the survival experiments were much higher than the measured O_2 consumptions, and thereby contributed to high control deaths.

When exposed to toxic concentrations of electrolytes, grouped *D. longispina* have a survival advantage over single animals (Fowler, 1931). The advantage, Fowler says, is due to lowered oxygen consumption per animal caused by accumulation of CO_2 in the medium. However, most of Fowler's experiments were conducted for very short time periods (10 hours or less); in those which ran longer than 10 hours, especially at higher concentrations, there tended to be a higher probability of single, rather than group, advantage. Over a longer time course, as in my experiments, possibly the CO_2 concentration in a small volume became high enough to be toxic to the animals, rather than to enhance survival. Accumulation of toxic waste products in small volumes was suggested by Warren (1900) and Woodruff (1911, 1914) (both in Fowler, 1931), as an explanation of harmful effects due to crowding. The possibility is further strengthened by the

fact that, in the present O_2 consumption experiments, which were conducted in large volumes and at low densities, there was virtually no death by 40 hours. Unfortunately, controlled measurements of pH were not made during either the survival or O_2 consumption experiments, so concentrations of CO_2 cannot be determined.

Another possible explanation for higher death rates of controls at the higher density is the feeding regime. Animals at 1 *Daphnia*/1 ml were not fed during the experiment; starvation could thus have accounted for a percentage of the deaths. At lower densities, however, animals were fed every 48 hours, and control values still dropped to about 40% survival. Probably the explanation for control deaths is a combination of factors inherent in the experimental design, with CO_2 toxicity, feeding regime, and stress due to confinement being important factors.

As previously stated, the interactions in any toxic response are complex. Our results leave unanswered several questions concerning the responses to dichromate of *Daphnia pulex* at various ages and densities. Precise information is needed on the responses of *D. pulex* under "normal" conditions, especially the following: oxygen consumptions at various ages and densities, effects of stress due to confinement, effects of various feeding levels on growth, reproduction, and oxygen consumption, and effects of varying levels of CO_2 on survival and oxygen consumption. Without such information it will be impossible to distinguish the effects of a toxin such as dichromate from the effects of other environmental factors.

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RÉSUMÉ

Les effets du polluant qu'est le dichromate de sodium ($Na_2Cr_2O_7$) sur la survie et la consommation d'oxygène de *Daphnia pulex* ont été étudiés pour différents âges et avec des densités variables. Avec une densité d'1 *Daphnia* pour 1 ml, et avec des concentrations supérieures à 0,10 mg/l, la mort survenait dans les 24 heures à chaque fois. Avec des concentrations de 0,01 mg/l et inférieures à ce taux, il n'y a pas de différence importante entre les taux de contrôle de mortalité et les animaux exposés au dichromate à raison de trois densités. Les taux de mortalité mesurés pour les deux âges soumis aux tests (6 jours et 12 jours) ne présentent pas de différence appréciable.

La mort tend à survenir plus tard avec des densités plus faibles de *Daphnia.* Ceci peut être dû à une consommation supérieure d'oxygène chez chaque animal, quand les densités sont plus faibles; il y aurait ainsi une acclimatation plus rapide à de faibles concentrations de substances toxiques.

Les consommations d'oxygène de *D. pulex* exposé à un taux de 0,01 mg/l de dichromate étaient

considérablement plus élevées que les valeurs de contrôle obtenues. Ces différences étaient dûes en partie à la présence d'une progéniture dans les poches incubatrices des animaux exposés au dichromate. Cependant, le développement plus rapide des jeunes chez les animaux exposés au dichromate était en lui-même probablement dû à la consommation d'oxygène accrue provoquée par l'exposition au dichromate.

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TABLE I

Effects of hexavalent chromium on microcrustaceans. (Adapted from: Water Quality Criteria, 1963)

Concentration of chromium, mg/l	Compound used	Type of organism	Remarks	Reference
0.016	$Na_2Cr_2O_7$	*Daphnia magna*	toxic threshold	Anon., 1950
0.05	?	*Daphnia magna*	killed in 6 days	Grushko, 1949
0.10	$Na_2Cr_2O_7$	*Daphnia magna*	toxic threshold	Anderson, 1944
0.10	Na_2CrO_4	*Daphnia magna*	toxic threshold	Anderson, 1944
0.51	Na_2CrO_4	*Daphnia magna*	toxic threshold	Fairchild, 1955
0.7	$K_2Cr_2O_7$	*Daphnia*	threshold effect	Bringmann and Kuhn, 1959
1.4	Na_2CrO_4	*Gammarus pulex*	total mortality	Saurent, 1956
0.1	$Na_2Cr_2O_7$	*Daphnia pulex*	95% mort. by 72 hr. (den. 1 D./ml)	present paper
0.01	$Na_2Cr_2O_7$	*Daphnia pulex*	75-85% mort. by 72 hr. (den. 1 D./ml)	present paper

MATERIALS AND METHODS

Daphnia pulex were removed from laboratory cultures and subcultured in baby-food jars containing 100 ml of *Daphnia* medium (for composition, see Appendix I); a density of 25-30 animals per jar was maintained. Young *Daphnia* were removed to separate containers within 24 hours of hatching. Animals were kept in a growth chamber at a temperature of 21°C ($\pm$ 0.5°C) and a 16L:8D photoperiod (light from 7 a.m. to 11 p.m.), and were fed an excess of the alga *Chlamydomonas moewusii.*

Survival

A stock solution of 100 mg/l $Na_2Cr_2O_7$ in *Daphnia* medium was prepared. Because of the tendency of chromium compounds to adsorb to the sides of the container (Standard Methods, 1965) a fresh stock solution was prepared before each experiment. Serial dilutions were made, and test animals within 24 hours of the same age were placed in appropriate amounts of the diluted ion. Numbers of animals surviving at each concentration were counted every 12 hours until all or nearly all of the animals were dead. An animal was considered dead when it was motionless on the bottom of the container and no respiratory movements could be discerned. Light and temperature regimen were the same as for subculturing.

An experiment was conducted to determine the effect of very high concentrations (compared with the 0.05 mg/l standard) of dichromate on survival. For this experiment, a stock solution of 1000 mg/l was prepared. Concentrations of 1000, 500, 100, 50, 10, 1, and 0.1 mg/l $Na_2Cr_2O_7$ in *Daphnia* medium were tested by placing 5 animals in 5 ml of each concentration. Because of the extremely rapid death rate at 10 mg/l and above (fig. 1), these concentrations were considered to be immediately lethal, and were not tested further.

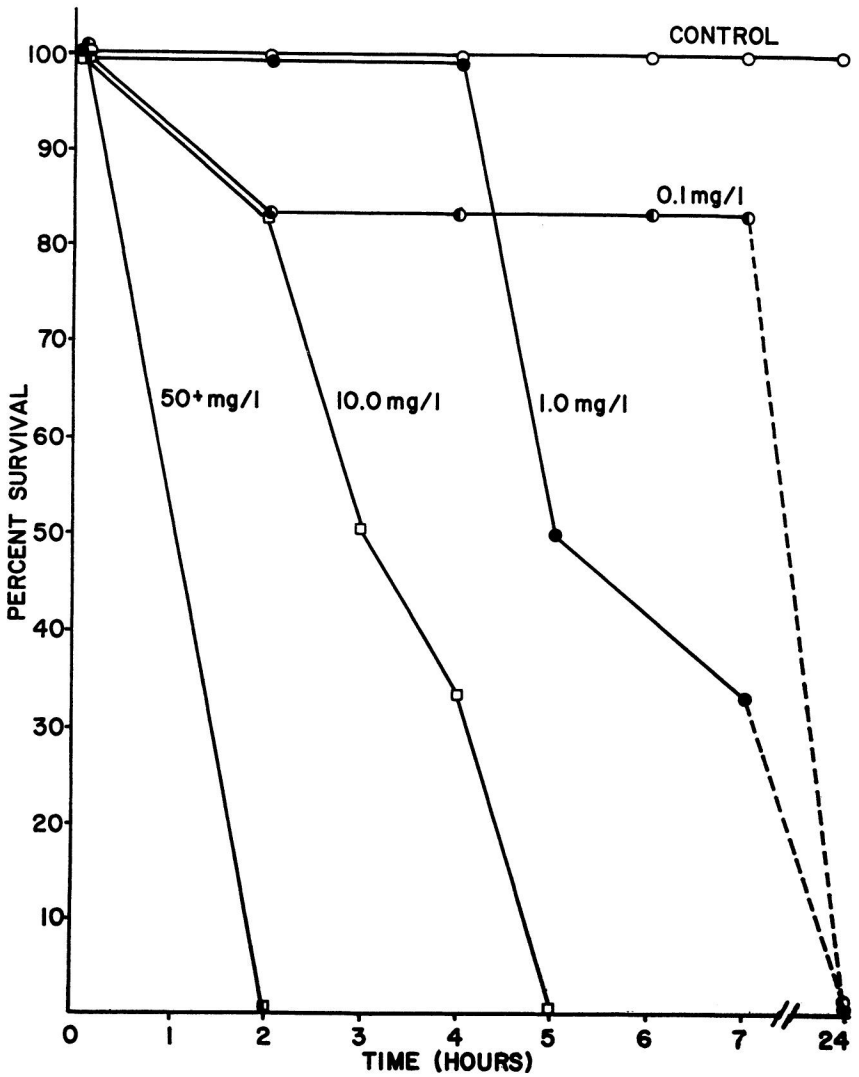

Fig. 1. Time course of survival of *Daphnia pulex* exposed to high concentrations of $Na_2Cr_2O_7$. At Hour O each group contained 5 animals.

In the first set of survival experiments, five concentrations of sodium dichromate were tested: 0.1, 0.05, 0.01, 0.005, and 0.001 mg/l. A control group in *Daphnia* medium was run with each experiment. Four experiments, totalling 40 animals per concentration, were conducted on 6-day-old animals; six experiments, totaling 40 animals per concentration, were done on 12-day-old animals. Because of difficulty in culturing enough animals, ages were tested separately. Readings on 6-day-old animals were taken every 12 hours from 0 through 72 hours; on 12-day-old animals, from 0 through 96 hours.

During tests, animals were placed in shell vials with a capacity of 10-12 ml. Animals were fed one hour before the beginning of, but not during, an experiment. All experiments began at a density of one ml of medium per animal; dead animals were not replaced.

The second set of survival experiments involved the interaction of animal density and dichromate concentration on survival. In each of six experiments, two concentrations of $Na_2Cr_2O_7$ (0.1 and 0.01 mg/l) and two densities (1 *Daphnia*/2 ml; 1 *Daphnia*/4 ml) were used. A control group in *Daphnia* medium was run at each density and concentration. All experiments were conducted on 6-day-old animals. Animals were fed before and every 48 hours during the experiment by leaving them in the original test solutions and adding three drops of concentrated *Chlamydomonas moewusii,* in *Daphnia* medium, to each vial. After 30 minutes they were removed to freshly prepared test solutions. Readings were taken every 12 hours from 0 through 96 or 108 hours. Data for the two densities are based on percentages of 24 (at 1 *Daphnia*/4 ml) and 57 (at 1 *Daphnia*/2 ml) animals surviving at each time period.

In all survival tests, percent survival was transformed to probit units (Finney, 1952); probits were then plotted against hours, and regression lines computed (figs. 2, 5, 6). Regression analyses began at the point on each time curve where survival began to decrease markedly. Comparison of homogeneity of regression coefficients was made using the Student's t-test (Steele & Torrie, 1960). Variation in degree of response among concentrations, densities, and ages was tested using the Kolmogorov-Smirnov two-sample test (Siegel, 1956). Absolute numbers of dead animals at each time interval were used for this determination.

Oxygen consumption

Oxygen consumption measurements were conducted on 6-day-old *Daphnia pulex* at two densities, 8 and 16 *Daphnia*/60 ml. *D. pulex* were placed in 60-ml glass-stoppered bottles. At each density there were four groups, each containing ten replicates. Replicates in the first two groups contained *Daphnia* in either aerated *Daphnia* medium or 0.01 mg/l dichromate solution. The other two groups contained only *Daphnia* medium or dichromate solution.

Samples were placed in a growth chamber (21°C, 16L:8D) and *Daphnia* were allowed to consume oxygen for 40 hours. Oxygen measurements were made with a Beckman oxygen analyzer. Oxygen consumptions were determined by subtracting the average of the bottles lacking *Daphnia* from the readings of the individual bottles containing *Daphnia.* Bottles were checked every eight hours for dead animals, but since few or no animals died, oxygen consumptions in all cases were figured on the basis of the original number of animals.

After oxygen consumptions were measured, lengths of *Daphnia* were determined from head to proximal end of tail spine. *Daphnia* were then placed on cover slips, dried for 24 hours in an oven at 60°C, and weighed on a Mettler microbalance. A faulty balance during part of the experiment, and loss of animals

during the drying process caused inaccurate weighings in some cases. Therefore, since length measurements were considered to be more accurate than weight measurements, the cube of the average length per *Daphnia* in each 10 replicates was used as an appropriate substitution for weight.

Readings of oxygen consumptions were made in mg/l and transformed into micrograms per 60 ml (μg/60 ml). Results were expressed as μg O_2 consumed/hour/animal and as μg O_2 consumed/hour/mm^3 of *Daphnia* tissue. Comparisons of controls and dichromate-exposed animals, and of densities at each concentration, were made using the Student's t-test (Steele & Torrie, 1960).

Dichromate adsorption

Because heavy metal ions, including dichromate, adsorb to the surface of the container (Standard Methods, 1965), an experiment was conducted to determine the rate of adsorption of various concentrations to the test vials. A stock solution of 100 mg/l was prepared. Standards of 1.0, 0.4, 0.1, 0.04, and 0.01 mg/l were made by serial dilution from the stock, and measured colorimetrically using a Zeiss PMQII Spectrophotometer at a wavelength of 540 mμ. The test concentrations of dichromate ion were 1.0, 0.1, 0.05, and 0.01 mg/l. Three vials containing 8 ml of dichromate in *Daphnia* medium at each concentration were measured every 12 hours from 0 through 72 hours. A new stock and a new set of standards were prepared at each 12 hour interval. The experiment was repeated three times.

Probably because of the very low concentrations measured, results of the adsorption experiments were extremely variable, and were not analyzed statistically. Because the measurable changes at these concentrations, while variable, were very slight, it is assumed that adsorption in the present experiments was negligible.

RESULTS

Survival

All survival curves of *Daphnia pulex* used in the first set of experiments are basically similar, being fairly linear over at least part of the time course, and in most cases indicating a slowing of mortality toward the middle of the time course (fig. 3: 36-48 hours; fig. 4: 48-72 hours). The slowing of mortality may simply be an artifact resulting from a fairly small sample size (40 animals per concentration), but its uniform occurrence suggests that it may be actual.

Death rates were high at all concentrations, including controls. Rate of death generally increased with increasing concentration (fig. 2). Differences between concentrations are illustrated in table II. There was little significance of age on rate of death.

The second set of experiments involved two concentrations (0.01 and 0.10 mg/l) and two densities (1 *Daphnia*/2 ml; 1 *Daphnia*/4 ml). At the higher density (fig. 5), survival rates of experimentals are nearly linear over the entire time course. At the lower density (fig. 6), however, very little death occurs from 12

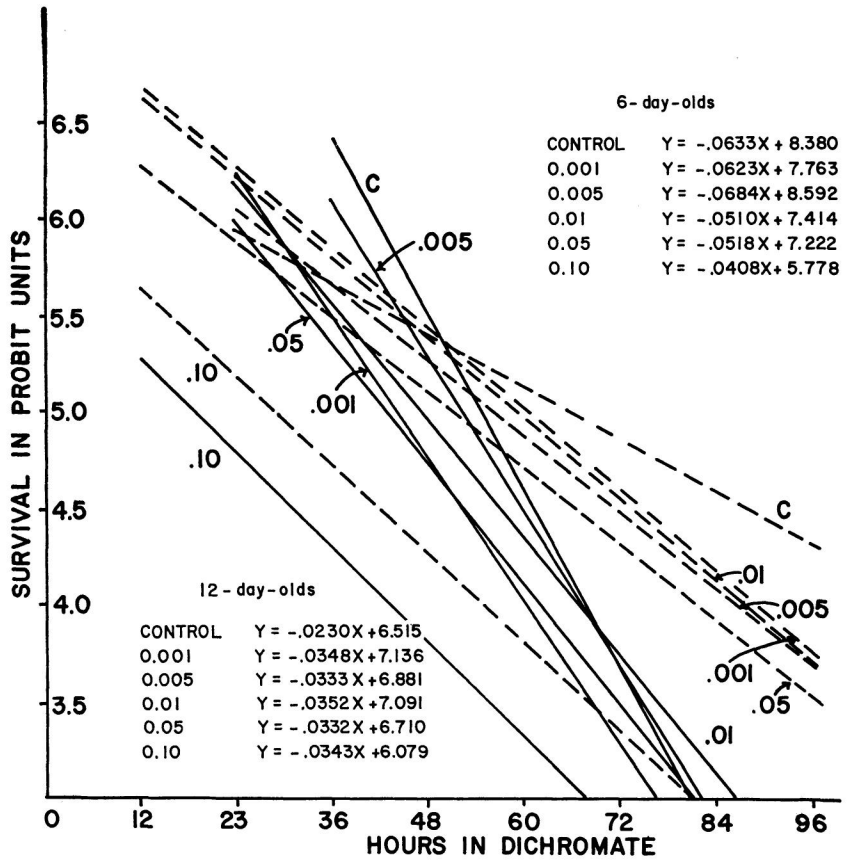

Fig. 2. Regression analyses of *Daphnia pulex* survival at various concentrations of $Na_2Cr_2O_7$. Solid lines refer to 6-day-old animals, broken lines to 12-day-old animals. Each line is based on the survival of 40 animals. Concentrations are in mg/l. Regression analyses for each curve are based on the part of the curve past the reflection point.

TABLE II

Kolmogorov-Smirnov two-sample test (Siegel, 1956) on *Daphnia pulex* at a density of 1 *Daphnia*/ml. The test, based on absolute numbers of dead animals, measures homogeneity of position of regression lines. Critical value of K_d for significance at 0.05 is 13.

a. 6-day-old *Daphnia pulex*.

Comparison	K_d	*Significance*
0.001 vs 0.10 mg/l	19/40	$p<.05$
0.01 vs 0.10 mg/l	16/40	$p<.05$
cont. vs 0.05 mg/l	14/40	$p<.05$
cont. vs 0.01 mg/l	12/40	n.s. (almost)
cont. vs 0.001 mg/l	10/40	n.s.
0.05 vs 0.01 mg/l	5/40	n.s.

b. 12-day-old *Daphnia pulex.*

Comparison	K_d	*Significance*
0.01 vs 0.10 mg/l	21/40	$p<.05$
0.001 vs 0.10 mg/l	20/40	$p<.05$
cont. vs 0.05 mg/l	12/40	n.s. (almost)
0.05 vs 0.01 mg/l	10/40	n.s.
cont. vs 0.001 mg/l	8/40	n.s.
cont. vs 0.01 mg/l	8/40	n.s.

c. 6-day-old vs 12-day-old *Daphnia pulex.*

Comparison	K_d	*Significance*
6- vs 12-day cont.	13/40	$p<.05$
6- vs 12-day 0.001	13/40	$p<.05$
6- vs 12-day 0.005	8/40	n.s.
6- vs 12-day 0.01	10/40	n.s.
6- vs 12-day 0.05	9/40	n.s.
6- vs 12-day 0.10	6/40	n.s.

to 48 hours (0.1 mg/l) and 12-24 hours (0.10 mg/l), after which the survival curve is linear. Although a lower density apparently delays the response of *D. pulex* to dichromate, over the linear portions of the range there is no significant difference in the death rate, either among concentrations or between densities (table III).

TABLE III

Use of Student's t-test (Steele & Torrie, 1960) for comparison of homogeneity of regression coefficients of *Daphnia pulex* survival against time at two densities.

a. Comparison of concentrations of $Na_2Cr_2O_7$ at a density of 1 *Daphnia*/2 ml.

Comparison	*df*	*t*	*Significance*
cont. vs 0.1 mg/l	10	.0986	n.s.
cont. vs 0.01 mg/l	10	−.4132	n.s.
0.01 vs 0.1 mg/l	12	.4728	n.s.

b. Comparison of concentrations of $Na_2Cr_2O_7$ at a density of 1 *Daphnia*/4 ml.

Comparison	*df*	*t*	*Significance*
cont. vs 0.1 mg/l	7	−.4046	n.s.
cont. vs 0.01 mg/l	5	1.0497	n.s.
0.01 vs 0.1 mg/l	8	−.3077	n.s.

c. Comparison of densities.

Comparison	*df*	*t*	*Significance*
cont. 1/2 ml vs 1/4 ml	6	.4521	n.s.
0.01 1/2 ml vs 1/4 ml	9	.0996	n.s.
0.1 1/2 ml vs 1/4 ml	11	.0946	n.s.

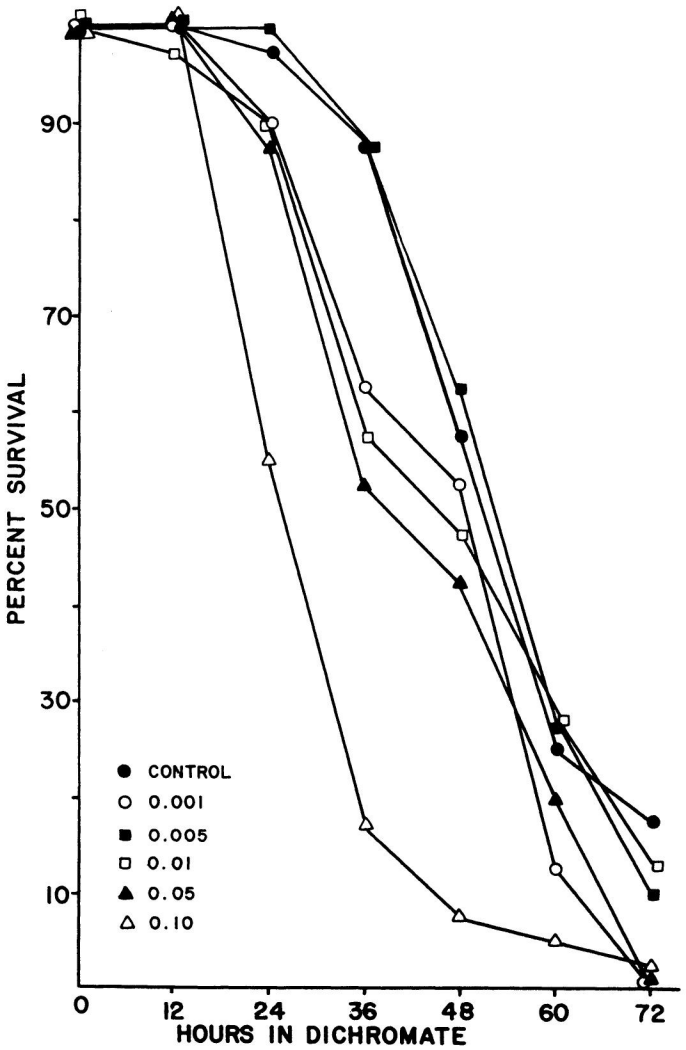

Fig. 3. Percent survival of 6-day-old *Daphnia pulex* exposed to various concentrations of $Na_2Cr_2O_7$. At Hour O, each group contained 40 animals, and the density was 1 *Daphnia*/ml.

Kolmogorov-Smirnov two-sample tests (table IV) indicate differences in positions of regression lines among concentrations at each density. In both cases, controls and the lower concentration (0.01 mg/l) were not significantly different, but both lived significantly longer than animals at the higher concentration (0.10 mg/l). Statistical comparison between densities was not possible because of variable sample sizes.

Oxygen consumption

Oxygen consumptions per animal in the dichromate-exposed group were approximately double those in the control group (table V, $p<.01$). Differences between

densities were less pronounced ($p<.05$ in controls) or not significant (dichromate). Low levels of significance probably result from the great variation among replicates, especially in the dichromate groups.

Calculating results as μg O_2 consumed/mm^3/hour decreases the variation due to size differences among animals, and therefore increases the degree of significance (table VI). Density differences in the dichromate groups are now apparent, and the differences between dichromate and control groups at 16 *Daphnia*/60 ml become highly significant.

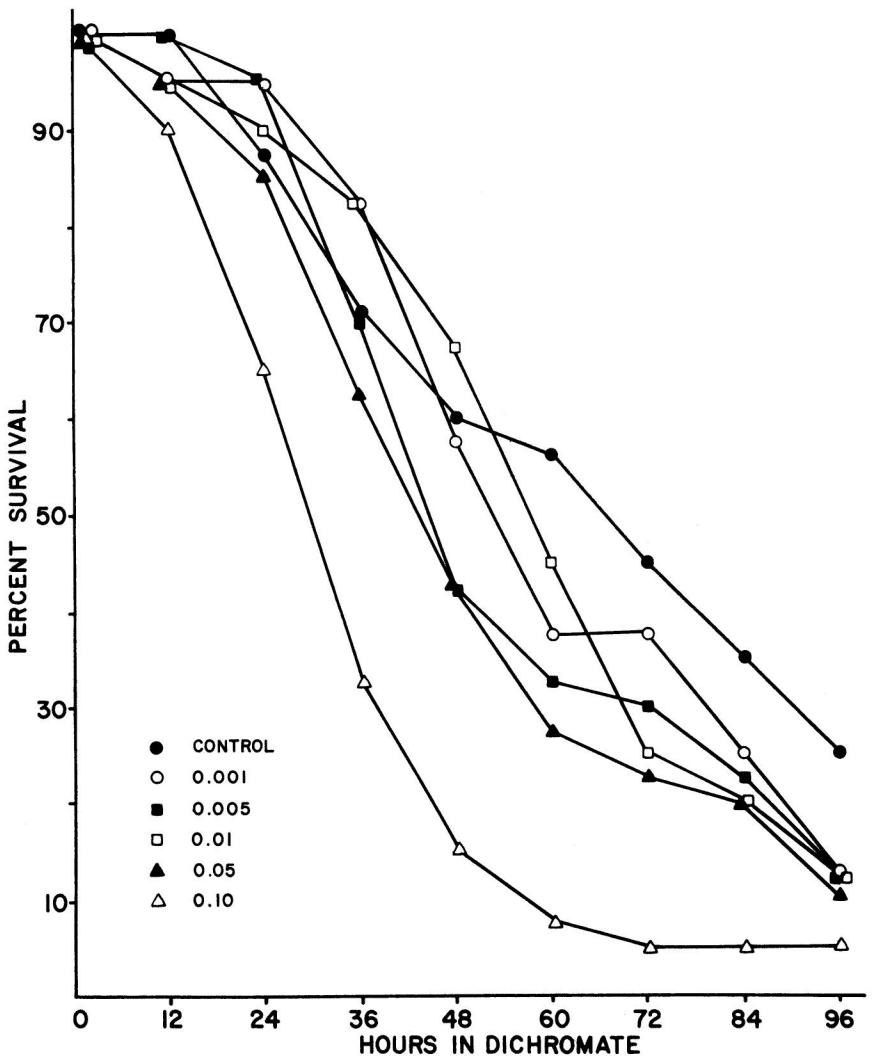

Fig. 4. Percent survival of 12-day-old *Daphnia pulex* exposed to various concentrations of $Na_2Cr_2O_7$. At Hour O, each group contained 40 animals, and the density was 1 *Daphnia*/ml.